Johansson G, Kopperschläger G and Albertsson PA (1983) Affinity partitioning of phosphofructokinase from baker's yeast using polymer-bound Cibacron blue F3G-A. *European Journal of Biochemistry* 131: 589–594.

Kopperschläger G and Birkenmeier G (1990) Affinity partitioning and extraction of proteins. *Bioseparation* 1: 235–254.

Tjerneld F, Johansson G and Joelsson M (1987) Affinity liquid–liquid extraction of lactate dehydrogenase on a large scale. *Biotechnology and Bioengineering* 30: 809–816.

Walter H and Johansson G (eds) (1974) *Methods in Enzymology*, Vol. 228, *Aqueous Two-phase Systems*. San Diego, CA: Academic Press.

Aqueous Two-Phase Systems

See **II/AFFINITY SEPARATION/Affinity Partitioning in Aqueous Two-Phase Systems**

Biochemical Engineering Aspects of Affinity Separations

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Introduction

Affinity separations are popular methods for the purification of biological molecules and other biological entities. They can readily be implemented on the laboratory scale but a number of additional factors have to be considered when these techniques are to be used for production purposes. Under these circumstances it is necessary to apply biochemical engineering principles to the design, scale-up and optimization of affinity separations. These topics are the subject of this article.

Selective interactions are exploited in affinity separations in order to achieve greater adsorbent selectivity for the desired molecule. Subtle differences in physical properties such as charge, size and hydrophobicity are often found to be insufficient for the required degree of purification in many separations of biological compounds. Many separations require the isolation of a minority component from a highly complex feedstock which may contain large amounts of similar compounds. As a consequence, it has been necessary to devise recovery flow sheets that consist of an extensive sequence of different steps – a sequence that may result in low overall yields and excessive costs. Hence affinity separations have been developed as alternatives to the more widely used separations based on ion exchange, hydrophobic interaction and size exclusion methods. Provided a ligand can be obtained which is truly selective for the desired component, it is possible to recover that component from a complex feedstock to a high degree of purity and in high yield. Typically the ligand is used in heterogeneous phase separations in which it is immobilized on to the surfaces of a porous solid-phase matrix material and employed in chromatographic and other adsorption techniques. Other approaches including the use of affinity ligands in selective precipitation and in modifying the phase selectivities in aqueous two-phase separations (ATPS) have been reported, but are not considered further here.

A variety of ligands with a wide range of molecular complexities have been developed for use in affinity separations and these are reviewed extensively elsewhere in this work. In many examples, duplication of the selective interactions that occur during the normal function of biomolecules have been exploited during such affinity separations; the affinity ligand is frequently one of the components of a recognition interaction. Examples include the recognition between an enzyme and its inhibitor or co-factor, or the highly specific interaction between an antigen and an antibody raised against it. Biomimetic molecules have been developed to mimic the recognition sites of more complex molecules, either by exploiting fortuitous interactions shown by readily available compounds (e.g. textile dyes) or as a result of the identification of new compounds either by studying the detailed three-dimensional structure of the target, or by the techniques of combinatorial synthesis. Selective molecular recognition can also be achieved without mimicking any naturally occurring

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biological interactions. A typical example would be in the use of immobilized metal ion affinity chromatography in the purification of proteins and peptides containing poly-histidyl sequences.

Although affinity separations are frequently considered to be highly selective, they are not always good at selecting between closely related variants of essentially the same compound, e.g. when changes have occurred that do not result in elimination of the molecular recognition of the ligand. For proteins, such changes include minor amino acid variations during synthesis, partial misfoldings, and the formation of dimers and higher oligomers. Under these circumstances, separations that exploit differences in the size or subtle differences in the surface characteristics of the molecules are called for; affinity interactions are not sufficient.

Affinity separations are mainly used in preparative techniques where the adsorbent is chosen to interact selectively with the desired product and hence to be used as a tool for its purification. An equivalent approach can be used in analytical techniques if the goal of the analysis is determination of the level of one or a group of closely related compounds. However, such an approach is comparatively rare as other, less selective, techniques when operated in a manner suited to the resolution of multiple components are able to yield, simultaneously, quantitative information about a larger number of components, even though such separations would not have sufficient throughput for use for preparative purposes. The approach that is adopted in this contribution is to introduce the considerations that are needed when affinity separations are to be used for preparative purposes at scales greater than that encountered in the laboratory (**Table 1**). Traditionally the approach and input of biochemical engineering to the optimization of bioprocesses is not considered important or necessary at the laboratory scale, where equipment and consumable costs are modest, and considerations such as yields, throughputs and batch cycle times are less important. The latter considerations become of much greater importance as the scale is increased through pilot to production procedures. However, much of what will be said is also applicable to the optimization of laboratory-scale procedures. The scale-up and optimization of affinity separations are characterized by features many of which are also of prime importance in the scale-up of other chromatographic methods and these should be considered in addition to the approach adopted here.

Table 1 Biochemical engineering aspects of affinity separations

Criterion	*Pertinent aspects*	*Implications*
Choice of affinity ligand	Selectivity	Purification achieved
	Strength of binding	Elution conditions
		Adsorbent logevity
	Chemical identity and origin	Cost
		Regulatory implications
Choice of immobilization material	Surface area	Contactor configuration and volume
	Mass transfer characteristics	Separation time
Position in process flow sheet	Reduction in overall number of steps	Process economics
	Feedstock composition	Adsorbent longevity
		CIP procedures
		Contactor design
	Feedstock volume	Bed volume
		Adsorbent kinetics
Process design	Optimization of separation performance	Laboratory-scale process development
	Choice of liquid-phase compositions, flow rates and stage durations	Computer modelling
Scale-up	Preservation of separation performance	Design heuristics and computer modelling
Monitoring and control	Measurement of levels of target molecule	Online process optimization
		Improved yield and productivity
	Detection of fault conditions	Manufacture to GMP standards
Process validation	Manufacture to GMP standards	Equipment installation and commissioning
		Process reproducibility and robustness
Economic	Minimization of costs	Adsorbent choice and longevity
	Competitiveness with alternative separation procedures	Liquid-phase selection
		Waste minimization, disposal and recycling

Position in Process Flow Sheet – Direct Recovery

One of the most important initial decisions regarding the adoption of an affinity separation technique in a recovery flow sheet is the selection of the point when that step should occur. Considerations of the expense and fragility of affinity ligands have often resulted in affinity separations being reserved for the later stages of a separation procedure, where, in general, the feedstocks are cleaner and the volume of liquid to be processed is less. Under such circumstances, affinity adsorbents would be expected to be able to be used for more cycles of operation, and each cycle of operation could be conducted in a smaller bed. Both these features would reduce process costs. However, this conservatism often results in underutilization of the potential resolving power of the affinity separation technique. The possibility of using a highly selective adsorbent for the capture and purification of an adsorbate from a very crude feedstock logically dictates that such a technique should be used at a very early stage in a separation protocol, thus eliminating the need for a series of less selective separation steps, each involving additional expense and a reduction of overall yield. However, the early stages of a separation protocol often involve feedstocks that contain particulates, including whole cells, pieces of broken cells and subcellular structures. The application of such feedstocks directly to packed beds results in the clogging of the bed arising from the capture of the particulates within the bed voids. Such feedstocks are typically pre-clarified by centrifugation or microfiltration, i.e. the use of techniques that not only may have expensive capital and running costs, but also may result in significant reductions in product yields. Recent advances in biochemical engineering have led to the development of a new technique to overcome the need for pre-clarification of the feed before application to an adsorbent bed. Expanded bed adsorption involves the use of beds with greater void volumes created by fluidizing the adsorbent beads in a stable manner as a result of upwards flow of liquid through the bed. Affinity ligands have been used with success in expanded bed adsorption techniques in order to achieve capture, concentration, clarification and purification in a single stage process.

Choice of an Appropriate Affinity Ligand and Immobilization to a Support

There are a number of factors that have to be considered when choosing an appropriate affinity ligand for use in affinity separations. One critical consideration is the selection of a ligand with appropriate affinity and selectivity towards the target molecule. Although the selection of ligands that form complexes with very low dissociation constants improves capture of adsorbate from feedstocks at low concentrations and permits extensive washing of the adsorbed complex to remove less tightly adsorbed impurities, it may prove difficult to achieve dissociation of the complex during the subsequent elution phase. Elution may need to be achieved as a result of major changes to the physical conditions of the irrigating buffer (e.g. pH, ionic strength) with the possibility of subsequent denaturation of the adsorbate and/or the ligand. In addition, there is the possibility that dissociation of the adsorbed complex will be slow as a result of low values of the rate constant of this step. When being used on a large scale, the cost of the affinity ligand and the number of cycles in which it can be used are also important considerations. Problems and costs associated with the manufacture of ligand in the quantities required to prepare large amounts of affinity adsorbents may become significant, together with any implications that the nature and the source of the ligand may have on the validation of the process. It may prove impossible to find ligand immobilization chemistries that totally prevent low levels of ligand leakage from the affinity adsorbent during all phases of the purification cycle. The use of affinity separations in the production of therapeutic products dictates the need for these processes to be operated in a manner that can be fully validated to comply with good manufacturing practice (GMP). The need for thorough sanitation as part of the inherent clean-in-place (CIP) procedures may necessitate exposure of the affinity ligand to harsh reagents or sterilization protocols not previously encountered during use of the technique in the laboratory, with a consequent increase in the likelihood of deterioration of the affinity adsorbent.

Once a suitable affinity ligand has been chosen, it has to be immobilized on to a suitable support in order to generate an affinity adsorbent. Considerations in the choice of a suitable support are common to those that would be used in other adsorption and chromatographic procedures. In general the use of porous particles has been the most popular in large-scale separations, although the benefits of using membrane materials in achieving fast mass transfer have been demonstrated in some small-scale systems. Important properties of a suitable support include:

- High surface area accessible by the target molecule per unit volume of matrix, to minimize the volume of the adsorbent needed for the separation.

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- Spherical particle shape and narrow particle size distribution to facilitate packing the bed to obtain optimal flow characteristics.
- Good mechanical properties of the matrix to resist compression and compaction in tall beds operated at high flow rates at high pressure drops and to resist attrition should removal of the adsorbent from the bed be necessary periodically for thorough sanitation procedures.

A wide variety of matrices, designed specifically for use in process-scale separations, are commercially available. In some cases, they may be purchased with popular, widely used, affinity ligands already covalently immobilized on their surface. Alternatively, base matrices may be available in a chemical form which facilitates customized covalent immobilization of more specialized ligands.

Operating Protocols, Equipment, Monitoring and Control

Affinity separations are carried out using equipment that is suitable for use in other adsorption and chromatographic procedures. In almost all cases this will involve the use of a packed bed of adsorbent, although the benefits of using expanded bed adsorption technology when processing particulate-containing liquids are also rapidly becoming apparent. These affinity procedures are operated in a batch mode with a number of sequential stages during each cycle of operation (adsorption, washing, elution, cleaning, re-equilibration). Reasons for the choice of such methods include the ease of automation and improved quality assurance over strirred tank adsorption procedures. Valves can be employed to ensure that the appropriate process solutions (feedstocks, buffers, eluents, etc.) are pumped on to the bed and that liquid fractions from the bed are diverted to appropriate vessels for collection. Typically these actions are under the control of an automated system which either follows a pre-programmed time sequence, or responds interactively to the features of the separation by exploiting information being received from sensors and monitors installed in the process.

In order to run an affinity separation optimally, it is necessary to be able to monitor the success of the separation as it is proceeding. Ideally, information is required on the levels of key components that accurately reflect the state of the separation. In affinity separations such components will include the adsorbate itself, the level of total protein and possibly the levels of key contaminants. The on-going development of biosensors able to provide continuous measurement of particular compounds will be of crucial importance in this area. Meanwhile, use is beginning to be made of techniques such as flow injection analysis, surface plasmon resonance sensors and rapid liquid chromatographic monitoring. These techniques yield much more specific information than is available from traditional flow measurement techniques such as spectroscopy (mainly used to measure the general level of protein) and pH and conductivity measurements whose contribution is restricted to monitoring the physical properties of the liquids flowing into and out of the column. The ability to be able to monitor online the levels of particular compounds enables optimization of the duration of the adsorption stage of the separation, terminating it when the level of the target compound begins to break through the bed without being captured. Such monitoring can also participate in the location of the target in the flow from the bed during elution.

Scale-up and Validation

One of the principal biochemical engineering considerations associated with affinity separations is to transform a successful laboratory separation procedure into a viable industrial unit operation. Packed bed separation procedures can be successfully scaled up to almost any extent provided certain rules are maintained and scale-up of affinity separations can also be achieved in a rational manner without dificulty. A suggested approach is:

- Ensure that the same adsorbent (including the chosen particle size) is used in both laboratory and large-scale procedures.
- Conduct investigations to determine the approximate dynamic capacity of the affinity adsorbent for the desired product and estimate the volume of the bed that will be needed to process the scaled-up batch size.
- Select a bed height of adsorbent that is compatible with the above volume. This will depend to a large extent on the diameters of commercially available columns, paying due regard to manufacturers' recommendations concerning minimum bed height to diameter ratios to achieve satisfactory flow distribution across the bed and maximum bed heights recommended to avoid excessive pressure drops and resultant bed compression.
- Optimize all stages of the separation in laboratory experiments using narrow columns of the same height as chosen for the full sized bed.
- Maintain the linear flow velocities and durations of each stage of the optimized laboratory process in the scaled-up procedure.

Adherence to these procedures should ensure that the characteristics of the separation and cycle time of the separation are maintained.

Before any downstream purification process can be used in the production of therapeutic agents it must be validated as part of the procedure to ensure compliance with GMP. For affinity separations, the issues are similar to those pertaining to any adsorption or chromatographic procedure. However, quality assurance issues associated with the source and origin of the affinity ligands may be of additional importance especially if these are molecules isolated from natural sources. Issues such as installation qualification and operational qualification and the associated documentation are dealt with in depth by Sofer and Hagel (see Further Reading).

Optimization

Two approaches to the optimization of affinity separations can be identified. The first 'practical' approach makes use of information and experience gained from experimentation, typically at the laboratory scale. Such an approach may be expensive and time consuming and may require the availability of substantial amounts of the target biomolecule, a situation that may be highly undesirable in the early stages of the development of a product with possible therapeutic value. The second 'theoretical modelling' approach is based on the philosophy that a complete understanding of the events that occur during an affinity separation can lead to optimization being achieved by computer-based methods. Such a situation is potentially cheaper and quicker, but requires confidence that the modelling approach adopted accurately describes the true situation. Sometimes it is necessary to combine the two approaches.

In addition to optimization of the choice of affinity ligand and the materials and methods for its immobilization, which have already been described above, a number of factors have to be considered when optimizing an affinity separation. These factors, which will have an influence on each other, include:

- Choice of flow rates. It is necessary to adopt a suitable compromise between fast rates to minimize purification time and slow rates to allow mass transfer and kinetic processes to occur. The nature of the matrix material chosen for the process and its resultant mass transfer characteristics strongly influence this consideration;
- Choice of irrigating liquid. The success of washing and elution procedures depends critically on the influence the liquid has on the strength of the adsorbent/adsorbate complex, particularly in relation to its effect on complexes of other compounds adsorbed specifically or non-specifically.
- Cutting the eluted peak. Most affinity separations achieve resolution and purification in the adsorption stage resulting from the specificity of the affinity ligand. However the use of 'group-specific' ligands may result in the adsorption of compounds other than the desired target. Under these circumstances attempts may be made to resolve the adsorbed species by use of a series of step changes in eluent composition and/or the use of a continuous gradient, and it is necessary to identify the portion of eluent containing the target compound. The cut may be made as a compromise between the yield and the degree of purification required.

Theoretical Modelling

A number of approaches have been adopted for the modelling of adsorption and chromatography operations in computer-aided process engineering. Some of these describe the features of the separation in gross, overall terms and essentially describe the mass balance over the process. Alternatively, other attempts have involved a detailed consideration of the details of such processes. One approach towards understanding the features that dictate the success and characteristics of an affinity separation has involved a detailed study of the nature and characteristics of the equilibrium and mass transfer processes for the adsorption/desorption of the adsorbate, and in some cases other key components in the separation. The nature of adsorbents adopted for use in practical affinity separations results in a totally thorough approach to modelling being complicated and impractical. Accurate analysis is frustrated by the presence of a distribution of particle diameters and pore characteristics, in addition to a non-homogeneous distribution of immobilized ligand throughout the interstices of the adsorbent. It has often been necessary to use approximate methods that overlook the latter complications. In general, the modelling of preparative chromatography is also difficult as a result of the need to consider the simultaneous adsorption of multiple species. However, the fact that affinity separations often involve the adsorption of only one or a few components can simplify the task. It must be remembered that although the selection and subsequent solution of a set of algebraic equations which describe the characteristics of an adsorption system can often be undertaken, it is also essential to obtain values for the parameters used in such equations which apply to the actual separation under

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Table 2 Strategies for optimizing the stages of affinity separations

Stage	*Criteria of efficiency*	*Achieved by*
Adsorption	Sharp breakthrough curves	Adsorbents with good kinetic properties (small particles, adsorption to outer surfaces) Low flow rates High inlet concentrations of adsorbate
	Selectivity	Appropriate choice of ligand
Washing	Maximum removal of contaminants, minimum removal of adsorbate	Appropriate choice of buffers
Elution (gradient)	Maximum concentration of product	Reversed flow direction Strong eluents Low flow rates
Elution (step)	Minimum denaturation of product	Weak eluents High flow rates

consideration. Commercial packages are now available which are reported to be effective for scale-up and optimization of affinity separations, although substantial improvements to a process can also be made on the basis of a qualitative understanding of the basic features of an affnity separation (**Table 2**).

It has already been pointed out that resolution in affinity separations is almost always achieved during the adsorption process and is less likely to be necessary during elution unless the specificity of the affinity ligand is low and multiple species have been adsorbed. Hence the majority of effort has been expended towards understanding the events that occur during the adsorption stage of the separation and is thus directed towards optimizing the duration of the adsorption stage to ensure that the potential adsorption capacity of the bed is utilized as far as possible, i.e. full use has been made of the costly immobilized ligand. Of particular importance is the correct assessment of the amount of adsorbate that can be removed from the feedstock during a cycle of operation. This requires knowledge of not only the maximum capacity of the adsorbent (q_m) but also the dissociation constant (K_d) of the adsorbed species. It is important to remember that the capacity of the adsorbent is governed by the nature of the adsorption isotherm, even when kinetic limitations allow the adsorbent to become loaded to equilibrium with the feedstock. In many cases, the equilibrium characteristics have been shown to be adequately described by a simple Langmuir isotherm, although the situation is certainly more complicated where more than one component can bind to the adsorbent. The shape of the adsorption isotherm is in most cases hyperbolic, with characteristics described by:

$$q = \frac{q_m c_A}{K_d + c_A}$$

Only in situations where the concentration of adsorbate in the feedstock, c_A, is of greater magnitude than the value of the dissociation constant of the adsorbed species (K_d) will the affinity adsorbent show equilibrium binding capacities as great as the maximum adsorption capacity (q_m). Indeed for values of c_A smaller than K_d, the equilibrium capacity of the adsorbent (q) decreases linearly with decreasing c_A and is given by

$$q = \frac{q_m c_A}{K_d}$$

This simple consequence of the law of mass action often accounts for the apparently low adsorption capacities that are observed during the development of affinity separations which are frequently mistakenly identified as a malfunction of the adsorbent. This point shows the importance of knowing the value of K_d of the selected ligand, but also the need to select an affinity ligand where the dissociation constant of the adsorbed complex with adsorbate is sufficiently low to ensure satisfactory capture of adsorbate from feedstock, particularly if attempts are being made to capture an adsorbate present at low concentrations.

Economic Considerations

Evaluation of the economics of the separation requires consideration of the fixed costs of the equipment (columns, reservoirs, pumps, valves, monitors, control system), and the running costs including the cost of the adsorbent, the chemicals used (including the need for large volumes of high quality water) and labour charges.

Although much attention is appropriately paid to the cost and longevity of the affinity adsorbent and its attached ligands, concentrating particularly on the number of cycles in which the adsorbent can be

used, due consideration must also be paid to the costs of all stages of the separation procedure. Such consideration should cover not only the basic costs of the chemicals employed, but also all costs associated with disposal and/or recycling of those chemicals after use. Some affinity procedures may involve specific eluents (e.g. enzyme co-factors) whose expense is greater than that of the simple strategies of changes in pH, ionic strength or dielectric constant used to elute many adsorbates. These economic considerations become of much greater importance in the design of process-scale procedures and are often overlooked at the laboratory scale.

Conclusions and Future Prospects

There are no technical barriers preventing the use of affinity separations in the production of biological molecules and entities. The biochemical engineering principles associated with the scale-up and optimization of affinity separations are well developed and the resultant conclusions are readily implemented. The conservatism surrounding their current use stems from the widespread lack of suitable affinity ligands. It is anticipated that novel molecules emerging from new techniques such as phage display technology and combinatorial chemistry will be excellent candidates for use of ligands in affinity separations. Affinity separations will be used in the purification of soluble biomolecules and also in the isolation of more complex species such as viruses, cells and other products for use in gene therapy. Affinity separations will therefore play an essential part in the preparation of future generations of therapeutic biotechnological products. Their adoption will result in the simplication and improvement of downstream processing flow sheets and will enable a rapid transition between discovery and utilization of these products.

See also: **I/Affinity Separation:** Covalent Chromatography; Dye Ligands; Rational Design, Synthesis and Evaluation: Affinity Ligands; Theory and Development of Affinity Chromatography.

Further Reading

Chase HA (1984) Prediction of the performance of macropreparative affinity chromatography. *Journal of Chromatography* 297:179.

Chase HA (1988) Optimisation and scale-up of affinity chromatography. In Jennisen HP and Müller W (eds) *Macromolecular Symposia 17*. Basel: Hüthig & Wepf Verlag.

Chase HA (1988) Adsorption separation processes for protein purification. In Mizrahi (ed.) *Downstream Processes: Equipment and Techniques*. New York: Alan R. Liss.

Chase HA (1994) Purification of proteins from feedstocks containing particulate material by adsorption chromatography in expanded beds. *Trends in Biotechnology* 12: 296.

Dean PDG, Johnson WS and Middle FA (eds) (1985) *Affinity Chromatography: A Practical Approach*. Oxford: IRL Press.

Harrison RG (ed.) (1994) *Protein Purification Process Engineering*. New York: M. Dekker.

Janson JC and Rydén L (eds) (1997) *Protein Purification: Principles, High Resolution Methods, and Application*. New York: Wiley.

Kline T (ed.) (1993) *Handbook of Affinity Chromatography*. New York: Dekker.

Ladisch MR (ed.) (1990) *Protein Purification: From Molecular Mechanisms to Large-scale Processes*. Washington, DC: American Chemical Society.

Scopes RK (1994) *Protein Purification: Principles and Practice*. New York: Springer-Verlag.

Sofer GK and Hagel L (1997) *Handbook of Process Chromatography: A Guide to Optimization, Scale-up, and Validation*. San Diego: Academic Press.

Subramanian G (ed.) (1995) *Process Scale Liquid Chromatography*. New York: VCH.

Wheelwright SM (1991) *Protein Purification: Design and Scale-up of Downstream Processing*. Munich: Hanser Publishers.

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Covalent Chromatography

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Introduction

Conventional affinity chromatography involves specific recognition of biomolecules such as antibodies and enzymes by immobilized ligands (antigens and inhibitors) usually by a multiplicity of non-covalent interactions. By contrast, the separation process in covalent chromatography does not require specific adsorptive binding and thus does not require knowledge of the structural determinants of the binding area of the component to be isolated. Instead, specificity relies on the nature of the chemical reaction of the chromatographic material with one or more components of a mixture. When complete specificity is